program Grant PG-11440. Address correspondence to Larry Fliegel, 417 Heritage Medical Research Center, University of Alberta, Edmonton, Alberta T6G 2S2,Canada.

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H. Wang, L. Fliegel, C.E. Cass, A.M.W. Penn, M. Michalak, J.H. Weiner and B.D. Lemire
University of Alberta
Edmonton, Alberta, Canada

Primers are Decisive for Sensitivity of PCR

ABSTRACT

A sufficient sensitivity of PCR is a prerequisite for its use in the diagnosis of infectious diseases. We have used PCR for detecting gene elements of Borrelia burgdorferi, *mycobacteria and* Bordetella pertussis. *With all these microbe groups, difficulties were encountered in achieving the demanded sensitivity with the primer pairs primarily selected. An extensive testing of various reaction parameters did not improve the sensitivity. Subsequently, we synthesized more primers derived from slightly different positions of the original target sequences. When the original and new primers were tested in possible combinations, some primer pairs reached 100-fold to 1000-fold higher sensitivity than the primary pairs. We conclude that in optimizing the sensitivity of PCR, more emphasis should be put on testing of several primer pairs than on the extensive screening of reaction parameters. Thus far, a trial-and-error approach has to be used, because there is no means to predict the sensitivity properties of a selected primer pair.*

INTRODUCTION

Polymerase chain reaction (PCR) is used in the diagnosis of many infectious diseases (3,7). To detect small amounts of microbial DNA among eukaryotic gene material, PCR has to be very sensitive and specific. It is well known that the efficiency of PCR depends on several reaction parameters, such as the annealing temperature and concentrations of magnesium, primers and polymerase (9,12). We have used PCR for the diagnosis of infections caused by *Borrelia burgdorferi*, mycobacteria and *Bordetella pertussis* (4,5, 8,10,11). Although various reaction parameters were extensively screened, the primer pairs primarily selected did not always allow a sensitivity sufficient for clinical diagnosis. In contrast, a dramatic improvement of the sensitivity was obtained by new combinations of primers, which were derived from slightly different positions of the original target gene sequences.

Table 1. Sequences of the Oligonucleotide Primers

Oligonucleotide	Sequence (5′–3′)
B. burgdorferi	
B4	(128) CTGCTGGCATGGGAGTTTCT (147)
B5	(857) TCAATTGCATACTCAGTACT (838)
WK1	(271) AAGGAATTGGCAGTTCAATC (290)
WK2	(560) ACAGCAATAGCTTCATCTTG (541)
FL7	(767) GCATTTTCAATTTTAGCAAGTGATG (743)
M. tuberculosis	
MV1	(579) GGCCAGTCAAGCTTCTACTCCGACTGG (605)
MV2	(1001) GCCGTTGCCGCAGTACACCCAGACGCG (975)
MV4	(953) CCCGACGTTCAACAGCGGGTC (933)
MV5	(693) AACAGGCACGTCAAGCCCACC (713)
B. pertussis	
BP1	(12) GATTCAATAGGTTGTATGCATGGTT (36)
BP2	(192) TTCAGGCACACAAACTTGATGGGCG (168)
BP3	(41) CGAACCGGATTTGAGAAACTGGAAAT (66)
BP4	(164) AATTGCTGGACCATTTCGAGTCGACG (139)

MATERIALS AND METHODS

DNA Preparation

The details of DNA extraction from the bacterial cells and the clinical specimens have been described previously (4,10,11). Serial dilutions (dilution factor: 10) of purified DNA extracted from *B. burgdorferi* (Catalog #35210; ATCC, Rockville, MD, USA), *Mycobacterium tuberculosis* (H37Rv) or *B. pertussis* (a clinical isolate) were used for the sensitivity assessments. For pertussis PCR, DNA was also extracted from seven nasopharyngeal swabs collected from a culture-confirmed pertussis case during nine consecutive days. The first three of the swabs grew *B. pertussis* by culture.

Oligonucleotide Primers

Thirteen oligonucleotide primers were used (Table 1). Five primers were derived from the gene encoding 41-kDa flagellin of *B. burgdorferi* (5,8,11); four primers from the gene encoding the 32-kDa secreted protein of *M. tuberculosis* (10); and four primers from the repeated gene element of *B. pertussis* (4). Oligonucleotides were synthesized by an automatic DNA synthesizer (Model 391 PCR-Mate™ DNA Synthesizer; Applied Biosystems, Foster City, CA, USA) based on phosphoamidite chemistry. Five primer combinations (B4-B5, B4-WK2, WK1-B5, B4-FL7 and WK1-FL7) were tested in borrelia PCR, two combinations (MV1-MV2 and MV4-MV5) in mycobacterial PCR and four combinations (BP1-BP2, BP1-BP4, BP3-BP2 and BP3-BP4) in pertussis PCR.

Polymerase Chain Reaction

The standard reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton®

X-[illegible] 200 μM deoxyribonucleotides (Pharmacia P-L Biochemicals, Milwaukee, WI, USA), 20 pmol of each oligonucleotide primer, 1 U of polymerase (DynaZyme™; FINNZYMES, Espoo, Finland) and purified DNA. The reaction volume was 50 μL, and a total of 40 cycles were carried out in a thermal cycler (HB-TR1; Hybaid Ltd., Middlesex, UK). For the borrelia PCR, the temperatures were 94°C for 1 min (denaturation), 50°C for 1 min (annealing) and 72°C for 1.5 min (extension); for mycobacterial PCR, 94°C for 1 min, 55°C or 65°C for 1 min and 72°C for 1 min; for pertussis PCR, 94°C for 1 min, 57°C for 1 min and 72°C for 1 min. After amplification, a 20-μL volume of the reaction mixture was run in a 1.5% or 2% agarose gel. After staining with ethidium bromide and destaining with water, PCR products were visualized and photographed under UV light.

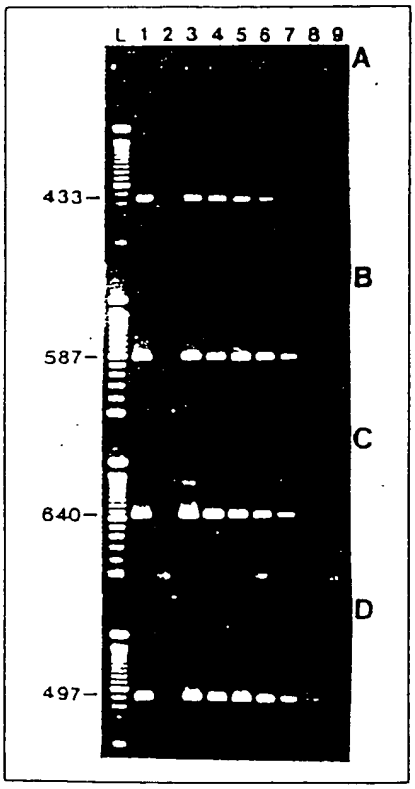

Figure 1. Amplification of purified *B. burgdorferi* DNA with four primer pairs targeted at the gene encoding 41-kDa flagellin. For all panels, lane L contains molecular weight markers; lanes 8 and 9, positive and negative controls; lanes 1–7, DNA serial dilutions (dilution factor [illegible]) of DNA extracted from *B. burgdorferi* [illegible] per reaction tube; lane [illegible]. The PCR protocol [illegible] described in Materials and Methods. (A) primer pair B4-WK2, (B) primer pair WK1-B5, (C) primer pair B4-FL7 and (D) primer pair WK1-FL7.

RESULTS

For borrelia PCR, the primary primer pair was B4-B5 (Table 1). With the standard PCR protocol, the detection limit by this primer pair was 300 pg of DNA (about 6×10^4 organisms) per reaction tube. An extensive screening of reaction parameters, including concentration of primers, magnesium, oligonucleotides and polymerase, was done to find out the optimal conditions. Further, a preincubation of PCR mixture at 96°C for 10 min was tested, as well as annealing temperatures of 42°C and 54°C. None of the tested conditions provided any improvement in the sensitivity of borrelia PCR, and in most of them the performance of PCR was even worse than in the standard conditions (data not shown).

After this unfruitful optimization of reaction parameters, three new primers (WK1, WK2 and FL7) were synthesized. The new primers and the original ones were tested in four combinations using the standard PCR protocol. The detection limits obtained by two of these pairs (B4-WK2 and WK1-B5) were one-tenth lower (30 pg per reaction tube) than that obtained by the original pair (Figure 1, A and B). One primer pair (B4-FL7) improved the sensitivity 100-fold (3 pg per reaction tube) (Figure 1C). The detection limit obtained by the fourth pair (WK1-FL7) was the lowest among these primer pairs (0.3 pg per reaction tube) (Figure 1D).

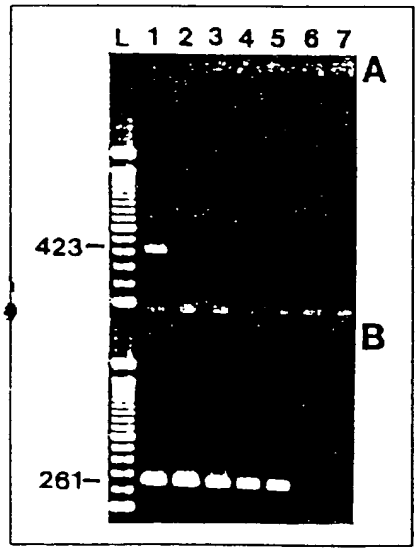

Figure 2. Amplification of purified *M. tuberculosis* DNA with two primer pairs targeted at the gene encoding the 32-kDa secreted protein of *M. tuberculosis*. For both panels, lane L contains molecular weight markers; lanes 1–6, serial dilutions (dilution factor 10) of purified DNA extracted from *M. tuberculosis* (lane 1, 5 ng per reaction tube, lane 6, 50 fg per reaction tube) and lane 7, negative control. The PCR protocol used was as described in Materials and Methods. (A) primer pair MV1-MV2 and (B) primer pair MV4-MV5.

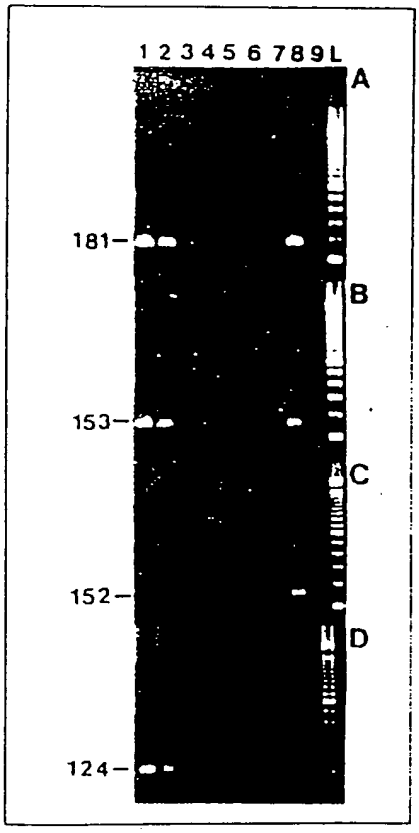

Figure 3. Amplification of purified *B. pertussis* DNA with four primer pairs targeted at repeated gene element of *B. pertussis*. For all panels, lane L, [illegible] molecular weight markers; lanes 8 and 9, positive and negative controls; lanes 1–7, DNA preparations extracted from seven nasopharyngeal [illegible] samples collected from a culture-confirmed pertussis case during nine consecutive [illegible]. Samples 1–3 were culture-positive. The PCR protocol used was as described in Materials and Methods. (A) primer pair BP1-BP2, (B) primer pair BP1-BP4, (C) primer pair BP2-BP2 and (D) primer pair BP3-BP4.

The primary primer pair in mycobacterial PCR was MV1-MV2. The detection limit by this primer pair with the standard protocol was 50 pg per reaction tube (Figure 2A). A limited screening of reaction conditions was performed. However, no improvement could be obtained by changing the reaction conditions (data not shown). Two new primers were synthesized and tested in one new combination (MV4-MV5). The sensitivity obtained by the new primer pair was 50 fg per reaction tube (Figure 2B), thus being 1000-fold better than that obtained by the original combination.

In pertussis PCR, the detection limit was 6.5 pg per reaction tube with primer pair BP2-BP3, 650 fg per reaction tube with BP3-BP4 and 65 fg per reaction tube with BP1-BP2 and BP1-BP4. The sensitivity differences between the primer pairs were clearly shown also in the analysis of the nasopharyngeal samples collected from a culture-confirmed pertussis case during nine consecutive days (Figure 3). Of the three primer pairs, BP2-BP3 gave positive result from the first two of these specimens (Figure 3C), BP1-BP4 and BP3-BP4 from the first four specimens (Figure 3, B and D) and BP1-BP2 from all seven specimens (Figure 3A). The first three of the specimens grew *B. pertussis* by culture.

Despite the dramatic sensitivity improvement achieved by using optimal primer pairs, the PCR assays remained specific to the target organisms. Borrelia PCR detected only *B. burgdorferi*, mycobacterial PCR detected only bacteria belonging to the genus *Mycobacteriaceae* and pertussis PCR detected only *B. pertussis*.

DISCUSSION

PCR is a very powerful method for diagnosis of infectious diseases, provided that sufficient sensitivity is achieved. The amplification efficiency of PCR depends on the concentration of the key reagents in the reaction mixture, the thermal cycle parameters and the primers. The reaction conditions can be optimized by using a titration approach (9,12). There are also certain rules that have to be obeyed in the primer design. The primers should not anneal with each other, and high G and C content and sequences forming hairpin loops should be avoided.

Our results suggest that primers are decisive for the sensitivity of PCR, and that there is no reliable means to predict the sensitivity achieved by a given primer pair. Some primer pairs, which have been designed taking into account the basic rules, do not work as efficiently as expected. An extensive search for optimal reaction protocol may be unfruitful with these primers. Our findings are in accordance with the previous observations (1,2). Campbell et al. used PCR with two primer pairs

selected from the DNA sequence of the *Chlamydia pneumoniae*-specific 474-bp *PstI* restriction fragment. Although all reaction parameters were analyzed in all possible combinations, one primer pair still detected fewer infectious units of *C. pneumoniae* than the other. Durigon et al. evaluated 19 primers combined into 16 different pairs in the PCR of human parvovirus B19 DNA. Although all of the primer pairs detected all virus strains, some did so with greater sensitivity than others. The reasons for such a difference in sensitivity between primer pairs remain unanswered. It is not clear whether the spatial configuration of template DNAs or primers play to some extent a role in causing the phenomenon. The recombinant AmpliTaq® DNA polymerase (Perkin-Elmer) may be helpful, because the enzyme may have a better performance than other thermostable DNA polymerase to perform PCR with certain templates that contain stable secondary structure (6).

We conclude that if a sufficient PCR sensitivity is not obtained with a primarily selected primer pair, an extensive search for optimal reaction conditions may not be justified. It may be less time-, money- and work-consuming to test new primer pairs derived from slightly different positions of the original target sequence.

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Address correspondence to Qiushui He, National Public Health Institute, Department in Turku, Kiinamyllynkatu 13, SF-20520, Turku, Finland. Internet: mviljanen@ktl.fi

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Q. He[1], M. Marjamäki[1], H. Soini[1], J. Mertsola[1,2] and M.K. Viljanen[1]

[1]*National Public Health Institute*
[2]*Turku University Hospital*
Turku, Finland